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Report

Optical Reversal of Halothane-Induced Immobility in *C. elegans*

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Summary

Volatile anesthetics (VAs) cause profound neurological effects, including reversible loss of consciousness and immobility. Despite their widespread use, the mechanism of action of VAs remains one of the unsolved puzzles of neuroscience [1, 2]. Genetic studies in *Caenorhabditis elegans* [3, 4], *Drosophila* [3, 5], and mice [6–9] indicate that ion channels controlling the neuronal resting membrane potential (RMP) also control anesthetic sensitivity. Leak channels selective for K^+ [10–13] or permeable to Na^+ [14] are critical for establishing RMP. We hypothesized that halothane, a VA, caused immobility by altering the neuronal RMP. In *C. elegans*, halothane-induced immobility is acutely and completely reversed by channelrhodopsin-2 based depolarization of the RMP when expressed specifically in cholinergic neurons. Furthermore, hyperpolarizing cholinergic neurons via halorhodopsin activation increases sensitivity to halothane. The sensitivity of *C. elegans* to halothane can be altered by 25-fold by either manipulation of membrane conductance with optogenetic methods or generation of mutations in leak channels that set the RMP. Immobility induced by another VA, isoflurane, is not affected by these treatments, thereby excluding the possibility of nonspecific hyperactivity. The sum of our data indicates that leak channels and the RMP are important determinants of halothane-induced general anesthesia.

Results and Discussion

In *C. elegans*, *unc-79*, *unc-80*, and *nca-2;nca-1* mutants each lack the worm orthologs of mammalian NALCN (sodium leak channel, nonspecific) and display hypersensitivity to halothane but normal sensitivity to isoflurane [3, 4]. In air, *unc-79*, *unc-80*, and the *nca-2;nca-1* mutants are "fainters" (i.e., they move normally for a few body lengths and then halt). These mutants also cease movement within 1 s of entering liquid medium [15]. We refer to the loss-of-function mutations of these genes collectively as *nca(lf)*. Their gene products are expressed exclusively in neurons [16, 17]. Mammalian NALCN, known to control the resting membrane potential (RMP) in mouse neurons [14], rescues the *nca-2;nca-1* double mutant (the two paralogs of NALCN) in *C. elegans* [16]. Remarkably, the anesthetic sensitivity and movement phenotypes of *nca(lf)* are conserved in *Drosophila* [3, 5]. The knockout of the NALCN channel in mice results in a hyperpolarized RMP in hippocampal neurons and neonatal death due to abnormal periodic breathing [14]. We also found that changes in mitochondrial complex I activity (such as in the mutant *gas-1*) increase sensitivity to volatile anesthetics (VAs) [18–20]. The present work attempted to join these two lines of work by identifying common features between the two groups of mutants.

Gene Expression in Halothane-Sensitive Mitochondrial Mutants

Ion channel pathway expression analysis was performed on complex I mutants in data sets previously generated (accession no. GSE9967) [21] using Gene Set Enrichment Analysis (GSEA). We studied three VA hypersensitive complex I mutants, *gas-1(fc21)*, F22D6.4 (RNA interference [RNAi]), and C34B2.8 (RNAi) (EC_{50}s 1.1%, 1.8%, and 2.0%, respectively), relative to three nonsensitive complex I mutants (RNAi) (Y45G12B.1, Y56A3A.19, and D2030.4; EC_{50}s 2.9%, 2.9%, and 3.0%, respectively) and wild-type (N2; EC_{50} of 3.2%) animals [19, 20]. Of ion channel gene cluster families analyzed, only the potassium channel superfamily showed significant upregulation in the anesthetic sensitive animals ($p = 0.00$; $q = 0.00$). Multiple nonion channel pathways were also altered and will be presented elsewhere. Gene level analysis revealed that the K2P leak channel subclass of this superfamily was most highly upregulated in the hypersensitive mutants (see Supplemental Information and Table S1 available online). Validation microarray analysis in an independent data set limited to *gas-1(fc21)* versus wild-type controls (accession no. GSE9967) [21] similarly showed upregulation by analysis of variance (ANOVA) analysis of gene expression for many potassium channel family members (data not shown). This upregulation of K2P channels may contribute to the hypersensitivity to VAs in mitochondrial mutants, as VAs can activate K2P channels [22] and hyperpolarize the RMP.

The Effects of a Potassium Channel Agonist

If hyperpolarizaton of the RMP by activation of a K2P leak channel mediates anesthetic response, a K2P channel agonist might be additive with VAs. We tested the effects of a potassium channel agonist, riluzole, on wild-type and mitochondrial mutants [23] (Figure S1). *gas-1* (anesthetic hypersensitive) had an increased sensitivity to riluzole (Figure S1A), whereas *mev-1* (normal anesthetic sensitivity) [24] had normal sensitivity (Figure S1A). When wild-type (N2) animals were exposed to 200 μM riluzole, sensitivity to halothane was increased (3.2% versus 1.9%) (Figure S1B). 200 μM riluzole also increased the sensitivity of *gas-1* to halothane (0.9% versus 0.3%) (Figure S1B). Since riluzole is also known to inhibit voltage-gated Na^+ channels and glutamate release [25], the

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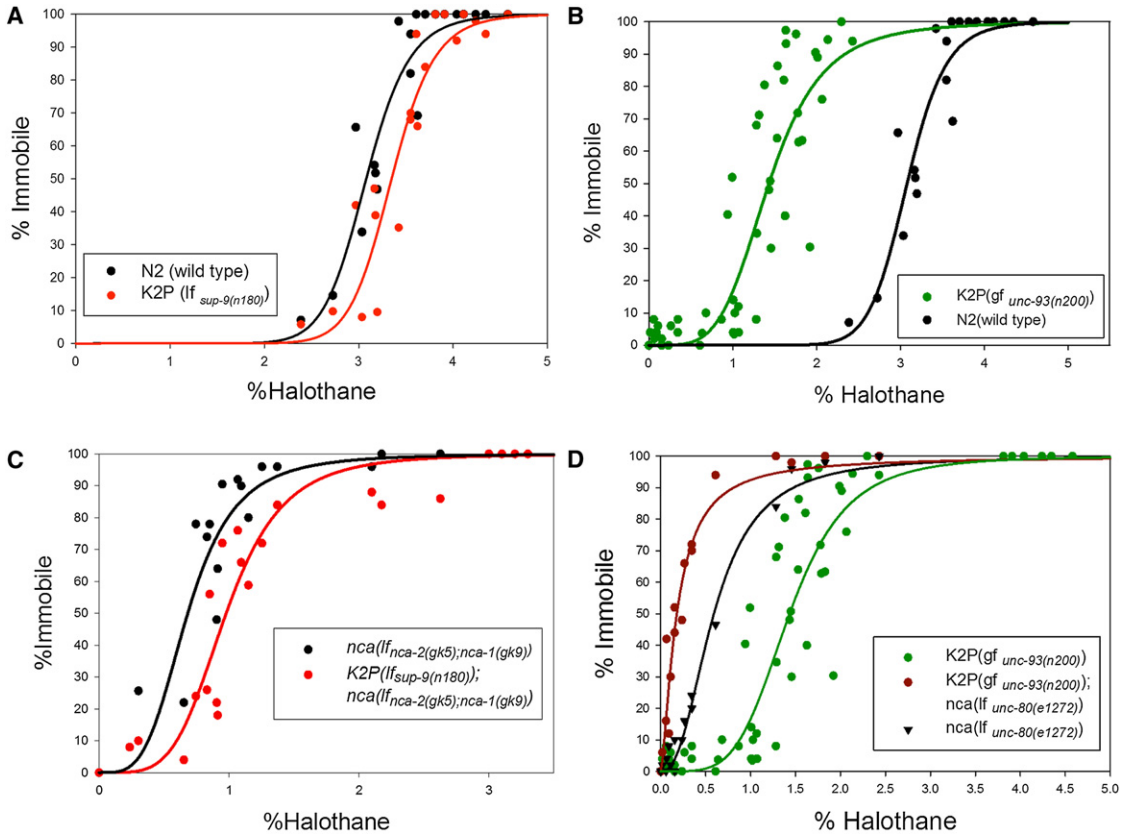

Figure 1. K2P Channels Alter Anesthetic Sensitivity in *C. elegans*

(A) *K2P(lf$_{sup-9(n180)}$)* shows a moderate resistance to halothane [N2, EC_{50} = 3.08% ± 0.06%, *K2P(lf),* EC_{50} = 3.35% ± 0.07%, $p < 0.006$].

(B) *K2P(gf$_{unc-92(n200)}$)* shows an increased sensitivity to halothane (EC_{50} = 1.43% ± 0.07%, $p < 0.0001$ versus N2).

(C) Loss of function of K2P channels partially rescues anesthetic sensitivity seen in the *nca(lf)* mutants [*nca(lf)* EC_{50} = 0.68% ± 0.05%, *K2P(lf);nca(lf)* EC_{50} = 0.98% ± 0.05%, $p = 0.0002$].

(D) Animals carrying both *K2P(gf)* and *nca(lf)* are extremely hypersensitive to halothane [EC_{50} = 0.18% ± 0.03%, versus *nca(lf)*, $p < 0.0001$ and versus *K2P(gf)*, $p < 0.0001$].

effects of riluzole may not be uniquely through activation of the K2P channels.

K2P Channels and Anesthetic Sensitivity

We predicted that mutants lacking K2P channels and therefore lacking a hyperpolarizing K^+ leak current would be resistant to VAs as seen in mice [6, 7, 9, 26, 27]. *C. elegans* possesses approximately 50 genes predicted to encode K2P channels [28]. One of these, *sup-9*, is the worm homolog that is most closely related to the mouse and human K2P channels TREK-1 (32% identity, clustalw alignment), TASK-1 (55% identity), and TASK-3 (58% identity). *sup-9(n180)*, a loss-of-function allele, has no phenotype in air [29] but is 8.7% resistant to halothane (Figure 1A).

UNC-93 is conserved in humans and thought to regulate the function of the K^+ channel formed by SUP-9 and SUP-10 [29–32]. We examined the anesthetic sensitivity of the gain-of-function (*gf*) alleles of *sup-10* and *unc-93* [collectively denoted as *K2P(gf)*]. *K2P(gf)* mutants are thought to conduct an increased K^+ current out of the cell [29]. The weakest of these *gf* alleles, *unc-93 (n200)*, is extremely sensitive to halothane with an EC_{50} of 1.43% ± 0.07% (Figure 1B). Other *K2P(gf)* mutants such as *sup-10(n983)* are also very hypersensitive to halothane (Figure S2, EC_{50} = 1.21% ± 0.007%). Similar results were seen with isoflurane (data not shown). The anesthetic hypersensitivity of *unc-93(gf)* and *sup-10(gf)* mutants can be suppressed by the *sup-9(lf)* alleles *n180* and *n1550n2174* [Figure S2, *sup-9(n180);unc-93(n200)* EC_{50} = 3.56% ± 0.17%, *sup-9(n1550n2174);sup-10(n983)* EC_{50} = 3.74% ± 0.06%]. These results suggest that K2P channel function is integral to anesthetic action in *C. elegans*.

We next examined how the K2P *gf* or *lf* alleles interacted with the loss of function of the NCA channels. The *K2P(lf)* mutation *sup-9(n180)* increased the EC_{50} of the *nca(lf)* mutant *nca-2(gk5);nca-1(gk9)* by 44% (Figure 1C). We next combined a *nca(lf)* mutation, *unc-80(e1272)*, with a *K2P(gf)* mutation, *unc-93(n200)* (Figure 1D). These double mutants move well in air but show a striking 17-fold reduction from the wild-type EC_{50} in halothane (0.18% ± 0.02% versus 3.1% ± 0.05%).

Optogenetic Reversal of Anesthesia

Cholinergic Neurons

If hyperpolarization of neurons in *nca(lf)* and *K2P(gf)* mutants causes the movement defects and halothane hypersensitivity, a depolarizing cationic current should rescue the defects. Channelrhodopsin-2 (*ChR2*) is a retinal dependent blue-light-activated cation channel from *Chlamydomonas reinhardtii* that has been used extensively in *C. elegans* to depolarize both muscle and neurons [33–35]. We expressed *ChR2* in

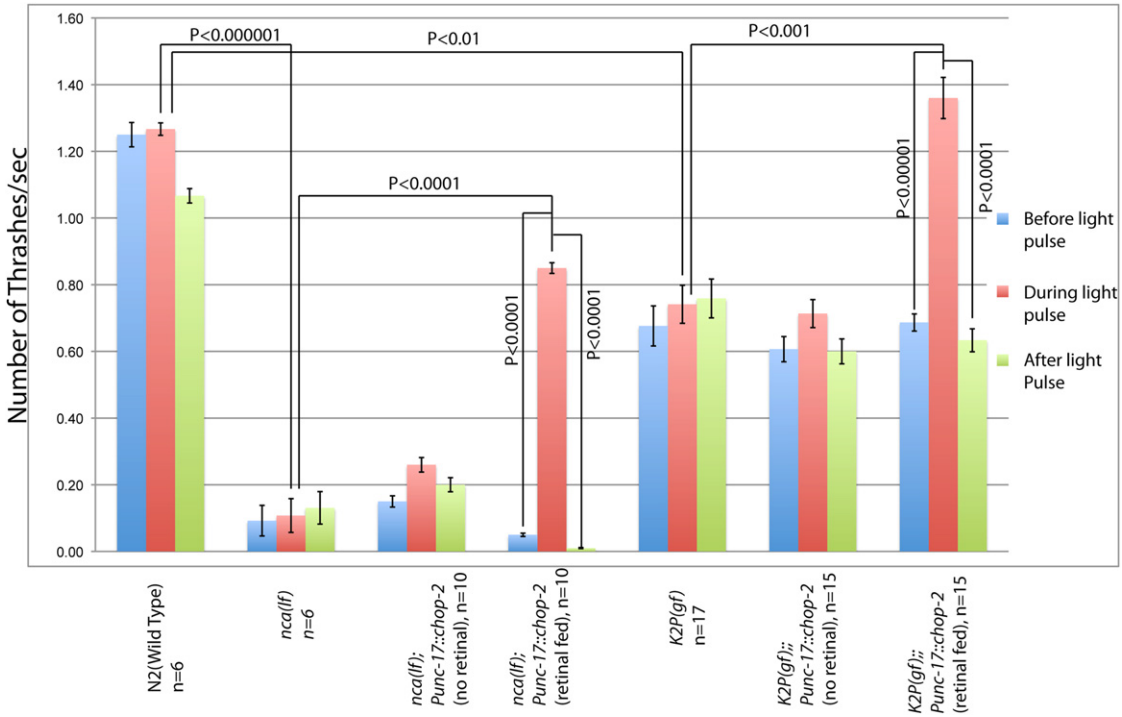

Figure 2. Rescue of *nca(lf)* and *K2P(gf)* Movement Defects

Swimming is moderately and severely impaired in *K2P(gf)* and *nca(lf)* mutants respectively, as measured by the number of thrashes/sec. Activation of channelrhodopsin-2 (*ChR2*) significantly improves swimming in retinal fed *nca(lf);Punc-17::ChR2* compared to the same worms before *ChR2* activation by blue light and when compared to blue-light-exposed non-retinal fed *nca(lf);Punc-17::ChR2* or *nca(lf)*. Upon cessation of blue light stimulation, these worms became paralyzed again. Activation of *ChR2* in retinal fed *K2P(gf);Punc-17::ChR2* worms lead to more thrashes compared to the non-retinal fed and non-blue-light-exposed controls. All bars represent the mean values of at least six measurements and are represented in the figure. Error bars represent SEM.

cholinergic neurons (under control of the *unc-17* promoter) in *nca(lf)* and *K2P(gf)* backgrounds.

nca(lf);Punc-17::ChR2 animals, like *nca(lf)* animals [17], were not able to swim in liquid medium (N2 = 1.25 ± 0.04 thrashes/sec, *nca(lf)* = 0.09 ± 0.28 thrashes/sec, *nca(lf);Punc-17::ChR2, no retinal* = 0.05 ± 0.005 thrashes/sec, Figure 2). When blue light was used to activate *ChR2* in the retinal fed *nca(lf);Punc-17::ChR2* animals, they were able to swim normally (Movie S1; Figure 2, *nca(lf);Punc-17::ChR2 retinal fed [during blue light]* = 0.85 ± 0.02 thrashes/sec). The fainting movement was also rescued upon *ChR2* activation by blue light (Movie S2). *K2P(gf)* mutants, although not as severely impaired as the *nca(lf)* mutants, were inhibited relative to N2 worms and improved upon *ChR2* activation (*K2P(gf);Punc-17::ChR2* animals [Figure 2]).

We next examined the anesthetic behavior of N2 animals expressing *ChR2* under *Punc-17*. We activated *ChR2* in all cholinergic neurons in *Punc-17::ChR2* animals anesthetized with halothane (Figure 3A, frames 1–3; Movie S3). Upon activation, the immobilized worms resumed sinuous motion (Figure 3A, frames 4–8; Movie S3) and promptly became immobile upon cessation of blue light illumination (Figure 3A, frames 9–12; Movie S3). Control worms not exposed to blue light remained immobile, as did control worms exposed to blue light but not fed retinal (Figure 3B; Movie S4).

The EC_{50} for the *Punc17::ChR2* transgenic worms was 4.3% ± 0.05% with retinal and blue light compared to 3.62% ± 0.04% without retinal and blue light (Figure 3B). The *Punc17::ChR2* strain was resistant to halothane compared to wild-type (EC_{50} 3.08% ± 0.06%) before *ChR2* activation by blue light. This may be due to an underlying conductance of a small leak current when *ChR2* is not activated by light and without the presence of retinal. We also examined whether immobility due to other anesthetics is reversed by *ChR2* activation. Surprisingly, isoflurane-induced immobility was not rescued by *ChR2* activation (Figure 3C) indicating these two anesthetics do not have identical mechanisms of action.

Activation of all cholinergic neurons could conceivably result in generalized animal hyperactivity that results in a reversal of immobility. Two points argue against this interpretation. First, activation of *ChR2* in cholinergic neurons of nonanesthetized worms did not increase movement but rather caused worms to become less mobile (Movie S7). Second, isoflurane-induced immobility was not rescued by depolarization of cholinergic neurons (Figure 3C). Activation of all cholinergic neurons may overwhelm the effects of anesthetics downstream of the neurons and reverse immobility. Therefore, we anesthetized *Pmyo-3::ChR2* animals, which express *ChR2* primarily in body wall muscle. These worms responded to blue light by contracting their muscles, but did not resume sinuous motion like the *Punc-17::ChR2* worms (Figure 3D; Movie S5). These data together argue against the possibility that the reversal of immobility by *ChR2*-based depolarization is a nonspecific effect of a generalized increase in the excitability of the nervous system. However, it is important to remember that there are considerable differences in the expression patterns of *nca* genes (pan-neuronal) [3, 16, 17], K2P channels (muscle, interneurons [29], and motorneurons

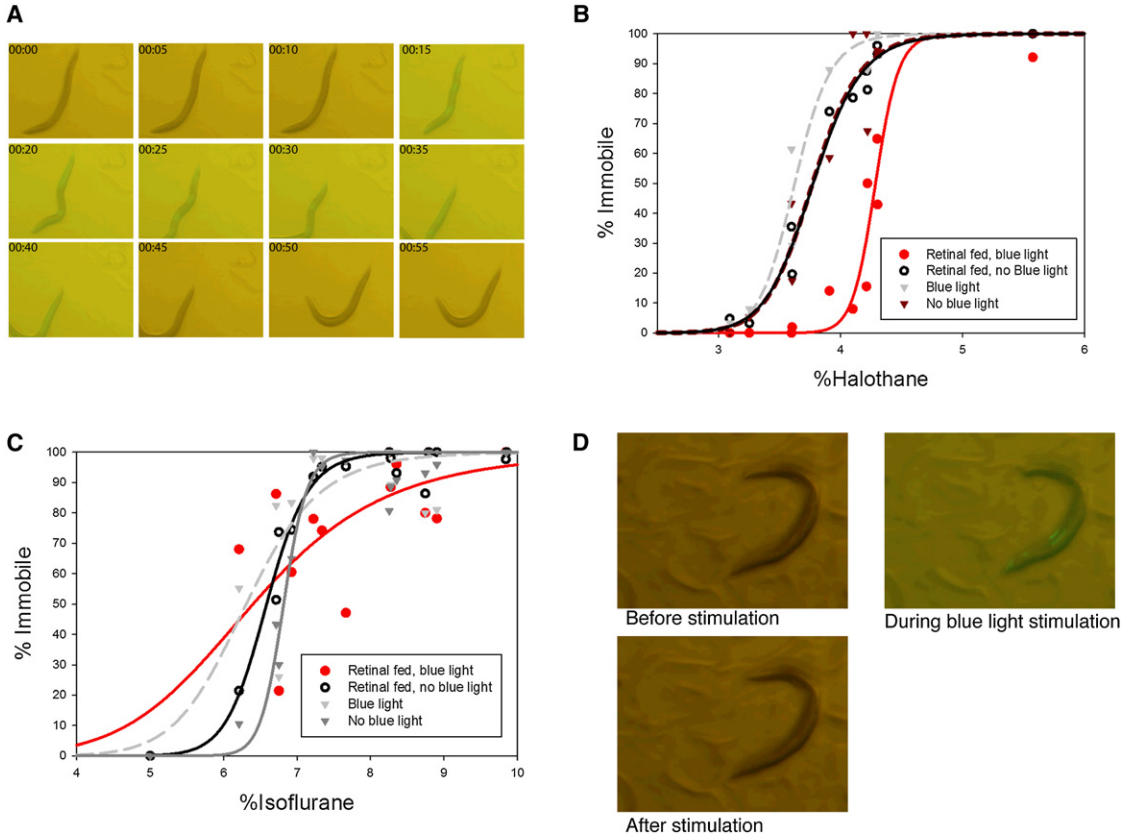

Figure 3. Reversal of Immobility in *C. elegans* Expressing Channelrhodopsin-2 in Cholinergic Neurons

(A) Stills taken every 5 s from a movie of a retinal fed *Punc-17::ChR2 C. elegans* immobilized by 4.85% halothane responding to blue light. Exposure to blue light causes the worm to resume sinuous motion again. Also see Movie S3.

(B) Dose-response curve showing anesthetic sensitivity of light-activated *Punc-17::ChR2* animals under halothane. EC_{50} is shifted to 4.29% ± 0.03% under blue light for retinal fed worms from 3.62% ± 0.04% for non-retinal fed control worms, $p < 0.0001$.

(C) Dose-response curve for the *Punc-17::ChR2* animals under isoflurane (EC_{50} retinal fed, blue light 6.4% ± 0.45% versus EC_{50} non-retinal fed, no blue light 6.82% ± 0.1%, $p = 0.3603$, not significant).

(D) *Pmyo-3::ChR2* worms fed with retinal when anesthetized under 4.8% halothane only contract their body wall muscles during blue light stimulation, resulting in no net movement.

[36]) and *unc-17* (cholinergic neurons [37]). Thus, we cannot ascribe all halothane effects only to cholinergic neurons.

Our model predicts that neurons in both wild-type and hypersensitive animals are similarly hyperpolarized when anesthetized by halothane. We hypothesized that the *nca(lf)* mutants would not only be rescued by *ChR2* activation at a concentration of halothane where all the *nca(lf)* mutants are immobile but also at a concentration where wild-type animals are immobile. To test this model, we determined whether anesthetic-induced immobility could be reversed in the *nca(lf)* hypersensitive mutants. Depolarization of the cholinergic neurons shifted the EC_{50} for halothane of the *nca(lf);Punc-17::ChR2* strain to 3.6%, well above the expected EC_{50} for wild-type *C. elegans* (3.1%), and 6-fold higher than the *nca(lf)* EC_{50} of 0.59% ± 0.05% (Figure 4A). Halothane-induced immobility was also reversed in *K2P(gf);Punc-17::ChR2* and *K2P(gf);nca(lf);Punc-17::ChR2* mutants by *ChR2* activation, at concentrations of halothane well beyond the EC_{100} levels for each mutant (Figure 4B).

GABAergic Neurons

As in mammals, gamma-aminobutyric acid (GABA) channels are the primary inhibitory channels in *C. elegans*. It has been suggested that VAs work by potentiating a hyperpolarizing chloride current through the $GABA_A$ channel [38, 39]. If this were the case, preventing GABA release in anesthetized worms should lead to some recovery of mobility. Expression of halorhodopsin (NpHR), a retinal-dependent chloride pump from *Natromonas pharonais*, can be used to hyperpolarize cells [33]. When NpHR was used to hyperpolarize all GABAergic neurons in halothane-anesthetized worms, no recovery of movement was seen. Even at concentrations of halothane close to the wild-type EC_{50}, control worms were more mobile than stimulated *Punc-47::NpHR* worms (Figure 4C). Therefore, the GABA channel is likely not a physiologically relevant target for halothane in *C. elegans*. However, there are many cys-loop ligand-gated chloride channels in *C. elegans*, and it is possible that other members of the superfamily contribute to anesthetic sensitivity.

Halorhodopsin Stimulation Increases Halothane Sensitivity

If the hypersensitivity seen in the *nca(lf)*, *K2P(gf)*, and the *nca(lf);K2P(gf)* double mutant results from neuronal hyperpolarization, then we must be able to mimic this hypersensitivity by hyperpolarizing neurons via other mechanisms. To this end, we anesthetized worms expressing halorhodopsin under the

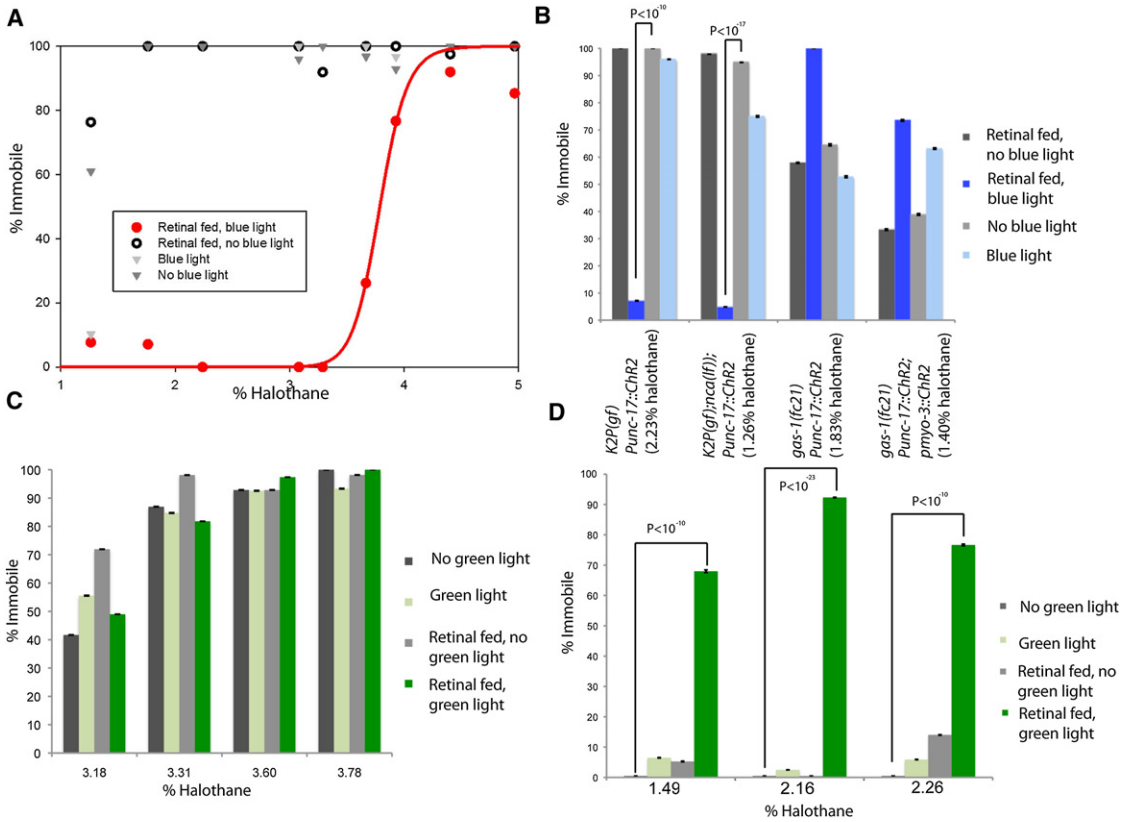

Figure 4. Mechanism of Anesthetic-Induced Immobility

(A) In *nca(lf);Punc-17::ChR2* depolarizing cholinergic neurons causes a significant rescue of anesthetic sensitivity (EC_{50} retinal fed worms 3.79% ± 0.04%, non-retinal fed and without blue light exposure, EC_{50} < 1%).

(B) *K2P(gf);Punc-17::ChR2* and *K2P(gf);nca(lf);Punc-17::ChR2* become mobile upon exposure to blue light in halothane [where the *K2P(gf)* and *K2P(gf);nca(lf)* are immobile]. *gas-1 Punc-17::ChR2,* and *gas-1 Punc-17::ChR2;Pmyo-3::ChR2* mutants did not become mobile when *ChR2* was activated in either cholinergic neurons or cholinergic neurons and muscle.

(C) In halothane anesthetized *Punc-47::halorhodopsin* worms, hyperpolarization of GABAergic neurons by halorhodopsin activation, did not reverse immobility at any concentration of halothane (at 3.18% halothane, no green light, no retinal = 41.6% immobile, green light, retinal fed = 48.9% immobile).

(D) In *Punc-17::halorhodopsin* worms, hyperpolarization of cholinergic neurons by halorhodopsin activation reduced the amount of halothane required for immobilization. Sixty-eight percent of animals were immobile at 1.49% halothane. Less than 7% of the non-retinal fed, and non-green-light-exposed animals were immobile at the same halothane concentration. A similar pattern is seen at 2.16% and 2.26% halothane.

See also Movie S6. All bars in (B)–(D) represent the mean values of at least 25 measurements. Error bars represent the SEM.

acetylcholine promoter (*Punc17::NpHR*). When halorhodopsin was activated by green light, these worms did not become immobile but instead phenocopied the *nca(lf)* mutants by fainting (Movie S6) and becoming hypersensitive to halothane (Figure 4D).

Failure to Reverse *gas-1* Hypersensitivity

Mutations that impair mitochondrial complex I function also cause anesthetic hypersensitivity in *C. elegans* [18, 19, 40]. This hypersensitivity was also seen in humans, because patients with complex I dysfunction were hypersensitive to VAs [41]. Mitochondrial mutants such as *gas-1* overexpress K2P channels, which can cause a hyperpolarized RMP, and account for halothane hypersensitivity. Interestingly, *gas-1(fc21) Punc-17::ChR2* did not resume movement with *ChR2* activation (Figure 4B). However, *gas-1* is expressed in all cells, including muscle [42]. Therefore, we examined the sensitivity of a strain of worms expressing *ChR2* in both cholinergic neurons and muscle (*gas-1 Punc-17::chop-2; Pmyo-3::chop-2*). Activation of *ChR2* in cholinergic neurons and muscle also failed to reverse immobility (Figure 4B). This suggests that the hypersensitivity to anesthetics in mitochondrial mutants either requires *ChR2* action in other neurons or involves additional mechanisms.

Conclusion

Artificial hyperpolarization of cholinergic neurons by halorhodopsin or depolarization by ChR2 significantly alters sensitivity of *C. elegans* for halothane-induced immobility. These results support the hypothesis that hyperpolarization underlies halothane's mechanism of action. The same is not true for isoflurane anesthesia, because ChR2 activation does not rescue movement of isoflurane anesthetized *C. elegans*. However, *nca(lf)* mutations are also not sensitive to isoflurane [3]. This provides further support of the hypothesis that different VAs function via distinct mechanisms, reminiscent of the difference in isoflurane and halothane protein binding shown by Eckenhoff et al. [43]. Our work does not show that either the K2P or the *nca* channels are direct targets of VAs. However, the data narrow the field of likely targets to those that can change membrane polarization in the presynapse, which includes both the K2P and NCA channels.

They also raise the possibility that modulating the RMP may serve as a reasonable mechanism for reversal of general anesthesia.

Experimental Procedures

Nematode Strains

C. elegans wild-type N2 (Bristol) and the strains *unc-93(n200)*, *sup-10(n983);sup-9(n1550n2174)*, *sup-10(n983)*, and *sup-9(n180)* strains were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). Other strains shared by the Jorgensen laboratory are detailed in the Supplemental Experimental Procedures. Channelrhodopsin-2::mcherry is abbreviated as *ChR2* in the manuscript. Halorhodopsin::GFP is abbreviated as halorhodopsin or NpHR.

Swimming Assay

A single worm was picked into 150 μl of S. Basal and allowed a minute to acclimatize. The number of thrashes was then counted for 10 s as described by Pierce-Shimomura et al. [15]. The number of thrashes for 10 s in the presence of blue light was counted and compared to the number after the blue light was discontinued. Results in Figure 2 represent the means of six experiments. Error bars represent SEM.

LED Illumination for ChR2 and NpHR Activation

A mightex LED controller (http://www.mightex.com, SLC-MA02-U) controlled duration and strength of LED illumination. A Luxeon rebel blue LED (~470 nm) was used for *ChR2* activation with 5 ms pulses at 100 Hz, at 1,000 mA [300 mA for the *nca(lf)* experiments]. A Luxeon rebel green LED (~530 nm) was used for halorhodopsin activation using a constant illumination at 1,000 mA. A red LED was used to illuminate the microscope field.

Procedures describing anesthetizing nematodes, RNAi treatment, and microarray analysis are included in the Supplemental Information.

Supplemental Information

Supplemental Information includes two figures, one table, Supplemental Experimental Procedures, and seven movies and can be found with this article online at doi:10.1016/j.cub.2011.10.042.

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